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Studies on Sexual Reproductive Characteristics of *Pteris vittata* L. in Soil Culture

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ABSTRACT.—In order to understand sexual reproductive characteristics of an arsenic hyperaccumulator, *Pteris vittata* L., in soil culture and provide valuable suggestions for its commercial production, two experiments were performed focused on how storage time of spores, collection time, substrate sterilization methods, and culture conditions affect sexual reproductive efficiency. Results demonstrate that spores of *P. vittata* remain at high viability after several month storage, can live through hot water and spores collected in winter have a higher viability than those collected in spring. Furthermore, light is indispensable for gametophyte growth of *P. vittata*. During the gametophyte phase and the early sporophyte phase, *P. vittata* demonstrated a wide adaptability to differing light intensities, but grew best under 40% shade. Additionally, it grew in sand though it prefers fertile substrates. A proper combination of light, substrate, and densities of spores and gametophytes promises efficient reproduction.

KEY WORDS.—collection time, light, *Pteris vittata*, sexual reproduction, spore viability, storage time, substrate

Pteris vittata L., commonly known as Chinese brake fern, is an arsenic hyperaccumulator, as well as a potential ornamental fern. Despite its great value in phytoremediation and gardening, it is not used widely. Numerous studies have been performed on this species. The mechanism of uptake and accumulation of arsenic by *P. vittata* has been studied in detail (Nie, 2006; Wang *et al.*, 2007; Yang *et al.*, 2007; Wei, 2008; Zheng *et al.*, 2008); its characteristics of gametophyte development have been documented (Zeng, 2001); and several regeneration methods, such as tissue culture and spore sterile culture, have been studied (Xu, 2006; Shi, 2008). However, much remains unknown, especially an understanding of the optimal conditions for the growth of gametophytes and sporophytes of *P. vittata* in soil culture. Compared with tissue culture and spore sterile culture, soil culture is simpler, cheaper and more convenient (Jiang, 2001), which could enhance the commercial production of *P. vittata*. Therefore, in order to understand its sexual reproductive characteristics in soil culture, two experiments were performed focused on how collection time, storage time of spores, substrate

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TABLE 1. Spore germination and gametophyte growth of the sowing group. The earliest green time was the earliest time when the substrate surface turned green after sowing; the earliest mature time was the earliest time when sexual organs emerged after sowing; the earliest time for juvenile sporophytes was the time when the first juvenile sporophyte emerged after sowing. The mean number of juvenile sporophytes of each replicate was recorded on 3rd September, 2008, when most juvenile sporophytes had at least three fronds.

Treatment	Collection dates	Sowing dates	The earliest green time*	The earliest mature time*	The earliest time for juvenile sporophytes*	Mean number of juvenile sporophytes (per replicate)
S1	2007.11.15.	2008.3.19.	14	62	74	120
S2	2008.1.16.	2008.3.19.	17	40	55	100
S3	2008.3.13.	2008.3.19.	17	62	101	37

* The number of days after sowing.

sterilization methods, and culture conditions affect the sexual reproductive efficiency of *P. vittata* in soil culture.

MATERIALS AND METHODS

Experiment I

Experiment I was conducted in an artificial climate box, aiming to reveal how collection time of fertile fronds, storage time of spores, and substrate sterilization with boiling water affect the sexual reproductive efficiency of *P. vittata*. Two groups, a sowing group and a no sowing group, were set. The no sowing group was set to examine sterilization efficiency. Based on three different collection times, three treatments (marked S1, S2, and S3) with four replicates per treatment were set in the sowing group. The no sowing group also included three subgroups (marked N1, N2, and N3) with four replicates in each subgroup. Therefore, there were 24 replicates in total. The experimental procedures are as follows:

Spore collection.—Fertile fronds of different plants of *P. vittata* were collected on three different days (Table 1) in South China Botanical Garden (Guangzhou, China), wrapped in paper bags, and dried at room temperature for four days to release spores. Then spores were collected in envelopes and stored at 4°C until used.

Substrate preparation.—Twenty-four transparent plastic cups of 6 cm in diameter with holes in the bottom were filled with sand, leaving space from the surface of sand to the cup mouth. After the sand was sterilized with boiling water, cups were sealed with plastic film to keep the sand away from pollution. Holes in the bottom of each cup helped prevent oversaturation.

Sowing and tending.—Spores weighing 0.05 g for each replicate of the sowing group by an electronic balance (Sartorius BS124S) were wrapped with filter paper, then sterilized using 1000× dilution of 50% Carbendazim powder and washed with hyperpure water three times, then made into a suspension.

Once the suspension was dropped onto the surface of sand evenly with a burette, the cup was sealed instantly with plastic film. No spores were sown in the no sowing group. After sowing, samples of the two groups were put in a tray filled with water. Then they were put in the artificial climate box. The cultivation conditions were: 18 $\mu mol \cdot m^{-2} \cdot s^{-1}$ fluorescent light for 15 hours, 25 °C, and 85%RH; dark for 9 hours, 20 °C, and 90%RH.

Observations.—To study the condition of spore germination and gametophyte development, random samples were taken weekly from the sowing group. Spores and gametophytes were mounted in water and observed under a light microscope (Zeiss Axioplan 2). Several parameters were recorded: the earliest green time, which was the earliest time when the substrate surface turned green after sowing; the earliest mature time, which was the earliest time when sexual organs emerged after sowing; the earliest time for juvenile sporophytes, which was the time when the first juvenile sporophyte emerged after sowing. The number of juvenile sporophytes of each replicate was counted when most juvenile sporophytes had at least three fronds. Meanwhile, quality of juvenile sporophytes was noted including the number and the size of fronds, and the number of pinnae. For the no sowing group, only the number of juvenile sporophytes of each replicate was recorded.

Experiment II

Experiment II was conducted in a plastic house, focused on how light and substrate affect the sexual reproductive efficiency of *P. vittata*. Five levels of sun light intensity and three kinds of substrate were laid out as follows: no shade (A), 40% shade (B), 85% shade (C), 96% shade (D), and dark (E), accomplished by different covers of black shade netting; sand (a), mixture of 50% sand and 50% farm soil (b; v/v), and mixture of 50% peat soil, 25% sand, and 25% farm soil (c; v/v). There were 15 treatments in total, with four replicates per treatment. The experimental procedures are as follows:

Spore collection.—Fertile fronds of *P. vittata* were collected on 23rd March, 2008 in the same place as Experiment I (Table 1), wrapped in paper bags, and dried at room temperature for four days to release spores. Then spores were collected in envelopes and stored at 4°C until used.

Substrate preparation.—Three kinds of substrate were put in 60 plastic garden pots of 12 cm in diameter, then sterilized with 500× dilution of Dexon powder and sealed as Experiment I for a week. Each tray under the pot was filled with water to ensure substrate inside was moist. One week later, plastic film was taken away and substrate was exposed directly to air for one week in order to let poisonous gas produced by the dilution of Dexon powder release gradually.

Sowing and tending.—Spores were weighed to 0.2 g for each replicate by an electronic balance (Sartorius BS124S) and wrapped with newspaper. After spores were sowed evenly on the substrate surface, the pot was sealed immediately with plastic film. Trays under the pots were kept full of water during this experiment. Meanwhile, microclimate condition under the five

different light treatments including temperature, relative humidity, and sun light intensity, was detected by a humidity-temperature meter (TES1364) and a light meter (TES1335) respectively at 2:30 to 3:00 in the afternoon every three or four days after sowing. Spores were sown on 18th April, 2008.

Observations.—Inspection of the condition of spore germination and gametophyte development with naked eyes occurred weekly. The number of replicates with at least 50% area green, the earliest time for juvenile sporophytes, and the number of juvenile sporophytes of each replicate were recorded in detail. For convenience, the number of juvenile sporophytes of each replicate was counted when the first juvenile sporophyte emerged. The earliest green time was recorded but not discussed in this experiment because algae or mosses turned the substrate surface green instead.

Results

Experiment I

Effect of storage time and collection time on the sexual reproductive efficiency of P. vittata.—The time when the substrate surface turns green is considered as the time when spores germinate here. According to the collection time and the sowing time (Table 1), storage times of spores were about four months, two months and one week, respectively. Spores stored longer did not germinate later. Spores with the longest storage time (S1) germinated earlier than those in S2 and S3, though the difference was not obvious. The substrate surface of S2 and S3 turned green simultaneously, though spores in the former was collected two months before sowing, and those in the latter collected just one week before sowing. Additionally, a longer storage time did not delay occurrence of sexual organs and juvenile sporophytes definitively. Sexual organs and juvenile sporophytes arose earliest in S2, but latest in S3. However, the longer spores were stored, the more juvenile sporophytes were born. The largest number of juvenile sporophytes was detected in S1, but only 37 juvenile sporophytes were found in S3 (Table 1), which was about one third of S2. In terms of collection time, spores collected in winter, such as on November 15th, 2007 and January 16th, 2008, germinated earlier and yielded more offspring than those collected in spring on March 13th, 2008.

Storage time and collection time together appear to influence the quality of juvenile sporophytes. Except S2, every replicate in S1 and S3 was covered fully with juvenile sporophytes. In addition, juvenile sporophytes from S1 or S3 were nearly the same size. In S1, sporophytes of each replicate had four to seven fronds with no more than two pairs of pinnae and the longest frond grew up to 3 cm long. In S3, sporophytes of each replicate had no or one to three fronds with no pinna and the longest frond grew up to 2 cm long. However, in S2 some sporophytes of one replicate had seven fronds while those of the remaining three replicates only had one to four fronds.

TABLE 2. Effect of light on the sexual reproductive efficiency of *P. vittata*. A, no shade; B, 40% shade; C, 85% shade; D, 96% shade; E, dark. The number of replicates with at least 50% area green was recorded 30 days after sowing; the earliest time for juvenile sporophytes was the time when the first juvenile sporophyte emerged after sowing; the total number of juvenile sporophytes of each treatment was recorded when the first juvenile sporophyte emerged.

Treatment	Number of replicates with at least 50% area green	The earliest time for juvenile sporophytes*	Total number of juvenile sporophytes
A	4	32	5
B	9	32	60
C	9	32	9
D	7	52	4
E	3	/**	0

* The number of days after sowing; ** no juvenile sporophytes emerged until 18th August, 2008.

Relationships among the earliest green time, the earliest mature time, and the earliest time for juvenile sporophytes.—Turning green earlier did not lead to an earlier occurrence of sexual organs. The sample in S2 became mature 22 days earlier than the other two treatments (Table 1), but S2 was not the first to turn green. S1, which turned green first, shared the same earliest mature time with S3, which turned green three days later than S1 (Table 1).The earlier sexual organs emerged, the earlier juvenile sporophytes arose. However, S1 and S3 did not produce sporophytes at the same time, though their sexual organs were born simultaneously.

Efficiency of sterilization with boiling water and its effect on spore viability.—Boiling water did not kill all spores in sand. No algae or mosses were detected in the sowing group or no sowing group. However, in one replicate each of N1 and N2, there were a small number of sporophytes born in N1 and N2 (N1 = 4 juveniles; N2 = 7 juveniles). Most sporophytes found in the no sowing group were recognized as *P. vittata*, and the rest died before they were big enough to be recognized.

Experiment II

Effect of light on the sexual reproductive efficiency of P. vittata.—Spores germinated under five levels of light intensity, even though those in the dark still germinated. However, the largest numbers of replicates with at least 50% area green were achieved under 40% shade and 85% shade. The earliest time for juvenile sporophytes was the same for treatment A, B and C, while sporophytes emerged 20 days later under 96% shade and no sporophytes were detected in the dark (Table 2). When the first juvenile sporophytes emerged, the largest number of juvenile sporophytes was achieved under 40% shade, 51 more than that under 85% shade (Table 2).

Effect of substrate on the sexual reproductive efficiency of P. vittata.—Spores germinated in three kinds of substrate. The largest number of replicates with at least 50% area green was achieved in the mixture of 50% peat soil, 25% sand,

TABLE 3. Effect of substrate on the sexual reproductive efficiency of *P. vittata*. a, sand; b, mixture of 50% sand and 50% farm soil; c, mixture of 50% peat soil, 25% sand, and 25% farm soil. The number of replicates with at least 50% area green was recorded 30 days after sowing; the earliest time for juvenile sporophytes was the time when the first juvenile sporophyte emerged after sowing; the total number of juvenile sporophytes of each treatment was recorded when the first juvenile sporophyte emerged.

Treatment	Number of replicates with at least 50% area green	The earliest time for juvenile sporophytes*	Total number of juvenile sporophytes
a	8	52	52
b	9	32	19
c	15	32	7

* The number of days after sowing.

and 25% farm soil, while only eight replicates with at least 50% area green in sand. Sporophytes arose simultaneously in substrate b and c, but 20 days later in sand (Table 3). However, the largest number of juvenile sporophytes was detected in sand rather than substrate b and c. Substrate c, the richest substrate, yielded the fewest juvenile sporophytes.

Comprehensive effect of light and substrate on the sexual reproductive efficiency of P. vittata.—Replicates in combination Aa, Ea, and Eb did not achieve 50% area green, and all treatments under 40% shade and 85% shade had the largest number of replicates with at least 50% area green. The earliest time for development of juvenile sporophytes differed among treatments. Sporophytes developed earliest in some combinations, such as Ab, Ac, Bb, Bc, Cb, and Cc; those in combinations Ba and Db developed later, and no sporopohytes were detected in combinations Da, Ea, Eb, and Ec. Combination Ba yielded the largest number of juvenile sporophytes, 43 more than combination Bb (Table 4).

Discussion

Green time and germination time.—In general, when a rhizoid emerges, the spore germinates (Zhang and Niu, 1999; Xu *et al.*, 2005; Xu, 2007). Based on our observation, when the gametophyte of *P. vittata* was only two to three cells long, chloroplasts were easily observed under the light microscope. In other words, the time when the substrate surface turns green is close to the time when spores germinate. Thus green time of the substrate surface is a reasonable and convenient indication of germination time.

Spore viability.—Results of Experiment I demonstrate that spores of *P. vittata* remain at high viability after several month storage and can live through hot water. High spore viability and strong heat-resistance ability of spores may contribute to the wide distribution of *P. vittata*, which spreads in the tropical areas and subtropical areas in Asia and America, occupies almost 25% area of China and even reaches Shaan'xi Province and Gansu Province, Northwest of China. Its distribution is growing. In 2006, for the first time, natural

TABLE 4. Comprehensive effect of light and substrate on the sexual reproductive efficiency of *P. vittata*. A, no shade; B, 40% shade; C, 85% shade; D, 96% shade; E, dark. a, sand; b, mixture of 50% sand and 50% farm soil; c, mixture of 50% peat soil, 25% sand, and 25% farm soil. The number of replicates with at least 50% area green was recorded 30 days after sowing; the earliest time for juvenile sporophytes was the time when the first juvenile sporophyte emerged after sowing; the total number of juvenile sporophytes of each treatment was recorded when the first juvenile sporophyte emerged.

Treatment	Number of replicates with at least 50% area green	The earliest time for juvenile sporophytes*	Total number of juvenile sporophytes
Aa	0	/**	0
Ab	1	32	4
Ac	3	32	1
Ba	3	52	51
Bb	3	32	8
Bc	3	32	1
Ca	3	72	1
Cb	3	32	4
Cc	3	32	4
Da	2	/**	0
Db	2	52	3
Dc	3	59	1
Ea	0	/**	0
Eb	0	/**	0
Ec	3	/**	0

* The number of days after sowing; ** no juvenile sporophytes emerged until 18th August, 2008.

populations of *P. vittata* were found on the city wall built in the Ming Dynasty, Nanjing, the capital of Jiangsu Province, East China. *P. vittata* was never reported in Jiangsu Province previously (Li, 2008). High spore viability, strong heat-resistance ability of spores, and wide distribution of *P. vittata* makes it a candidate for inclusion in gardening and phytoremediation.

It is commonly known that the longer storage time is, the later spores germinate (Pan *et al.*, 2007). However, in Experiment I, a longer storage period of spores did not delay germination and formation of sexual organs and sporophytes of *P. vittata*. This is distinct from the study on *Osmunda japonica* Thunb., which showed that given the same sowing density, spores stored longer tended to geminate later and led to later formation of gametophytes and sporophytes (Deng, 2004). This is because spores of the two genera are different. Spores of *Osmunda* have chlorophyll while those of *Pteris* do not, and the spore coat of *Osmunda* is so thin that its spores tend to dry out but the spore coat of *Pteris* is thick enough to resist drying.

Data from Experiment I shows that spores of *P. vittata* collected in winter have a higher viability than those collected in spring. This may be due to the increased activity of microbes in the spring. It is reported that microbes can reduce viability of spores and obstruct formation of gametophytes and sporophytes (Simabukuro, 1998). Microbes are rich under natural conditions. Spores used in Experiment I were exposed to microbes during the process from

TABLE 5. Sun light intensity, relative humidity and temperature under five different light levels.

Treatment	Sun light intensity ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Relative humidity (%)	Temperature (°C)
A	217.587±154.9299	88.25769±9.9522	29.96154±3.131242
B	158.1323±85.7517	84.31923±11.7896	30.56154±3.453324
C	37.02845±22.91277	83.71538±12.65758	30.43077±3.442245
D	10.89365±7.42368	84.96538±13.11077	30.09615±3.402542
E	0	87.61538±10.71748	30.03462±3.365771

fertile frond collection to spore collection, so some spores may have been contaminated by them before sterilization and thus had low viability. Due to the cold and dry weather in winter, spores collected during that time might be less affected by microbes than those collected in the warm and wet weather of spring. This could be the reason that spores of *P. vittata* collected in winter geminated earlier and yielded more offspring than those colleted in spring. Therefore, collecting spores in dry and cold weather ensures a high viability of spores, especially for *P. vittata*. Additionally, in order to kill microbes effectively before sowing, proper sterilization methods for spores and substrate are necessary.

Growth habits of gametophytes and juvenile sporophytes.—Data from Experiment II indicates that light is indispensable for gametophyte growth of *P. vittata*. However, spores could germinate in the dark in Experiment II. Spores of *Woodwardia radicans* (L.) Sm. also germinated in the dark during wet storage at 20 °C (Quintanilla *et al.*, 2002), but spores of *Osmunda cinnamomea* L. var. *asiatica* Fern. did not germinate in the dark (Jiang, 2001). This could be associated with chlorophyll. As mentioned above, spores of *Osmunda* have chlorophyll while those of *Pteris* and *Woodwardia* do not. Thus spores of *Osmunda* need light to germinate, while those of the other two genera do not.

Results of Experiment II imply that at the gametophyte phase and the early sporophyte phase, *P. vittata* has a wide adaptability of light intensity, but it grew best under 40% shade and most gametophytes under no shade died before the birth of sporophytes. This suggests that adequate shade is necessary for the well being of gametophytes of *P. vittata* despite being a hardy fern that likes brightness. Indeed many ferns need careful tending, similar to *P. vittata* at the gametophyte phase and the early sporophyte phase (Kong, 2000; Huang, 2006).

Different levels of light intensity lead to different sexual reproductive efficiencies of *P. vittata*. This is strongly connected to the microclimate under different covers of black shade netting. Combining the data about sun light intensity, relative humidity and temperature (Table 5) and the total number of juvenile sporophytes of each treatment (Table 2), a conclusion can be drawn that gametophytes and juvenile sporophytes of *P. vittata* prefer bright conditions with high moisture and mild temperature.

Besides a wide adaptability of light intensity, *P. vittata* also has a strong adaptability from poor substrate to rich substrate and could regenerate itself by spores in sand. This is why it can be found on sidewalks, building crevices, and nearly every habitat with exposed limestone and forms to several populations on the city wall in Nanjing (Li, 2008). However, it seems to prefer fertile substrate as the most gametophytes developed in the mixture of peat soil, farm soil and sand in Experiment II. Thus rich soil is preferable for a high reproductive efficiency of *P. vittata*.

When the first juvenile sporophytes arose in Experiment II, the largest number of juvenile sporophytes was achieved in substrate poor in nutrition, such as sand, rather than substrate rich in nutrition, such as the mixture of 50% peat soil, 25% sand, and 25% farm soil. This might be connected with the time counting the number of juvenile sporophytes. Given enough time, the number of juvenile sporophytes in substrate rich in nutrition would probably increase and even surpass that in substrate poor in nutrition. Therefore, a more reasonable time should be selected to count the number of juvenile sporophytes and more parameters should be set to examine effect of substrate on sexual reproductive efficiency of *P. vittata*. The physical structure of substrate could influence the number of juvenile sporophytes as well (Pan *et al.*, 2007). Given that the substrate was always saturated with water in Experiment II, due to the loose structure in sand, there might be more liquid water in sand than in the mixture of peat soil, farm soil and sand, so sperm of gametophytes cultured in sand could reach archegonia more easily. This is consistent with the conclusion that liquid water is critical for ferns at the gametophyte phase in sexual reproduction though nutrition is badly needed after sporophytes are born (Bao *et al.*, 2000; Sheffield *et al.*, 2001). Therefore, a mixture of sand and other materials in a proper proportion and enough fertilizer are indispensable for a high efficiency of sexual reproduction of ferns.

Besides culture conditions, densities of spores and gametophytes are factors that determine an optimal number of juvenile sporophytes. For instance, in Experiment II the largest number of replicates with at least 50% area green was achieved in the mixture of 50% peat soil, 25% sand, and 25% farm soil 30 days after sowing (Table 3), but the smallest number of juvenile sporophytes was found in those same conditions. Besides the improper time counting the number of juvenile sporophytes and the physical structure of the mixture, a density of spores or gametophytes that was too high may have resulted in lack of nutrition, an unbalanced proportion of antheridia and archegonia or something else (Liu and Liu, 2001), though a higher density of gametophytes contributes to more antheridia and more sporophytes for some species (Raghavan, 1989; Pan *et al.*, 2007). Further study should be performed to reveal how densities of spores and gametophytes affect the number of juvenile sporophytes and what proper densities of spores and gametophytes are for the commercial production of *P. vittata*.

The information provided in this paper certainly will be useful for the commercial production of *P. vittata*. However, further study remains to be performed as our study is focused on the early growth of *P. vittata*.

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